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# Negative modulation of alkaline phosphatase and creatine kinase by homobrassinolide

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## Abstract

Homobrassinolide is a plant hormone implicated in plant growth and development. Its effect on animal metabolism was less known to date. We have investigated its effect on the marker enzymes such as alkaline phosphatase and creatine kinase in selected rat tissues-brain, heart, liver, kidney, skeletal muscle and testis. Homobrassinolide was administered (66 and 330ng/ Kg body weight) intradermally in male albino wistar strain rats and changes in alkaline phosphatase and creatine kinase activities were measured. An overall reduction in both the enzyme activities occurred within 2hr of administration with few exceptions. The reaction rate constants for the enzyme activities were in the order $10^{-7}$ mM/min for alkaline phosphatase and $10^{-3}$ mM/min for creatine kinase. Time course studies indicated a decrease in enzyme activities as a function of time. Elevated hemoglobin content correlated with rise in erythrocyte number. Blood glucose level decreased by a percentage of 15.7 and 21.7 compared to control with the administration of 10µg and 50µg homobrassinolide respectively. Serum cholesterol content showed 15% decrease and 25% increase compared to control following 10µg and 50µg homobrassinolide administration. We conclude that homobrassinolide inhibited both the enzymes in the tissues and produced erythrocytosis, leukocytosis and hypoglycemia, while cellular phosphorylation status remained principally affected by this oxysterol in rat. Even though the physiological and pathological significance of these observations is not clear, it is suggested that 28-HB enriched diets may not be appropriate for higher energy related work activities.

**Keywords:** Alkaline phosphatase; Creatine kinase; Homobrassinolide; Oxysterol; Phosphorylation; Rate constant.

## Introduction

Homobrassinolide is a plant hormone, which belongs to brassinosteroid group. Brassinosteroids synthesized in plants exhibited potential to integrate various aspects of growth and regulation. In extremely low concentrations (0.01 – 0.1ng/gm fresh weight) the Brassinosteroid isoform 28-Homobrassinolide (28-HB) promoted seed germination, flowering [1], protein, DNA and RNA polymerase synthesis [2] in plants. Two marker enzymes namely Alkaline phosphatase and Creatine kinase plays important role in cell metabolism were studied. Alkaline phosphatases (ALP) are a large family of dimeric zinc-containing glycoproteins [3] present in all organisms. ALP is an allosteric enzyme [4]. ALP is known to dephosphorylate nucleotides on their 5' and 3' positions and proteins/organic molecules on their

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specific phosphates. L-aminoacids (L-Phe, L-Trp and L-Leu) reportedly inhibited ALP activity uncompetitively [5]. Creatine Kinase (CK) is a phosphorylating enzyme of significance in muscle contraction [6]. Under anaerobic conditions creatine phosphate is a precursor form of chemical energy used for ATP production [7]. Elevated serum CK activity due to the CK-MB isozyme is an important diagnostic tool for acute myocardial infarction [8].

Perturbations affecting these enzyme activities in the animal tissues signal alteration to metabolic processes involving them. Such perturbations were therefore indicators of cell stress. Brassinosteroids exhibited structural similarities to animal steroid hormones. Endogenous oxysterols reportedly affected cholesterol homeostasis [9] in normal animal cells. Homobrassinolide was found to reduce lipid peroxidation and elevate antioxidant defense [10]. They employed cellular nuclear receptors to communicate their biological potency. Dietary oxysterols can therefore be suspected of inducing metabolic stress in animal cells. Investigations were therefore directed to elucidate the effect of the oxysterol 28-HB, on the ALP and CK marker enzyme activities in male wistar rat tissues.

## Materials and methods

Male albino wistar strain rats (8-10 weeks, 150-170gm) were used for the study. para-Nitrophenyl phosphate, ADP and $NADP^+$ were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Phosophocreatine was obtained from Himedia, Bengaluru, India. The brassinosteroid isoform homobrassinolide (28-HB) was obtained from M/s Godrej Agrovet Ltd, Mumbai, India. Glass distilled water was used for preparation of all reagents.

Rats were organized into three groups (one control and two treated groups) with 6 rats each in a group. Ethanol was used as solubilizing vehicle for 28-HB. Ethanol was injected into normal rats and was maintained as control. Animals were administered 66ng and 330ng / Kg body weight of 28-HB (corresponding to 10µg/ml and 50µg/ml 28-HB) separately, intradermally which are maintained as treated groups. These concentrations were determined based on the minimal dose of this phytohormone required to elicit a biological response in the rat tissues.

The animals were handled as per the guidelines of the CPCSEA. Tissues (brain, heart, liver, kidney, skeletal muscle and testis) were surgically excised 2hr after 28-HB injection from anaesthetized rats and were washed twice in ice cold 1.15% KCl and homogenized to give 10% w/v. The tissue homogenates were then centrifuged at $4^oC$ in a refrigerated high speed centrifuge at 10,000 x g for 10 min. The supernatant was used for the measurement of ALP and CK enzyme activities and for total protein content.

Alkaline Phosphatase enzyme activity was assayed by the method of Bessey et.al [11], employing para–nitrophenyl phosphate as a substrate. The colour developed due to the formation of the product para-nitrophenol, was measured in a spectrophotometer at 410 nm. The intensity of colour generated was taken as a measure of the alkaline phosphatase enzyme activity. One unit of activity was defined as the amount of enzyme required to liberate free phosphate from one micromole of para-nitrophenyl phosphate at pH 10.5 and at $37^oC$.

Creatine kinase enzyme activity [12] was measured using a coupled reaction, employing phosphocreatine as a substrate. The change in absorbance due to the formation of NADPH during the initial 5min of the reaction was spectrophotometrically measured at 340nm. One unit of enzyme activity was defined as the amount of enzyme that converted one millimole of phosphocreatine to creatine per minute at pH 6.7 and at $37^oC$. Protein content of the homogenized samples was measured by the method of Lowry et al [13].

The time course study of ALP and CK enzyme activities was carried out by assaying both the enzyme activities at 0, 30, 60, 90 and 120min following ethanol administration to rats. Similarly the enzyme assays were carried out for 10µg 28-HB and 50µg 28-HB administered rats as well. Rat kidney homogenate 10,000 x g supernatant preparation was used for estimating ALP activity, and skeletal muscle homogenate 10,000 x g supernatant preparation was used for estimating CK enzyme activity due to the prominence of these two tissues for the enzymes under investigation.

Blood analysis was carried out using rat blood collected by cardiac puncture from anaesthetized (anaesthetic ether) animals. 1.5% EDTA was used as

an anticoagulant. Hemoglobin content was estimated by the acid hematin method [14]. RBC and WBC cell counts were measured by the method of John et al [15]. Glucose content in blood was measured using Asatoor and King method [16]. Serum cholesterol level was estimated by the method of Zak et al [17]. Statistical evaluation was done using ANOVA. Data in the tables represent Mean ± S.E.M of three independent determinations.

## Results

The present study showed a overall decrease in ALP and CK specific activities following 2hr administration of 28-HB (Tables – 1, 2) with few exceptions. ALP enzyme activity was found increased in 10µg 28-HB treated rat skeletal muscle tissue by 63% and in 50µg 28-HB treated rat testis by 3% compared to control.

**Table 1. Effect on ALP activity in rat tissue homogenates 2hr after administration of 28-HB.**

| | **Brain (IU/mg) x $10^{-3}$** | **Heart (IU/mg) x $10^{-3}$** | **Liver (IU/mg) x $10^{-3}$** | **Kidney (IU/mg) x $10^{-3}$** | **Skeletal muscle (IU/mg) x $10^{-3}$** | **Testis (IU/mg) x $10^{-3}$** |
|---|---|---|---|---|---|---|
| **Control** | 1.6±0.0 | 3.4±0.2 | 0.4±0.0 | 25.5±1.3 | 2.8±0.2 | 5.7±0.3 |
| **28-HB (10µg)** | 0.9±0.0* ↓49% | 3.2±0.1 ↓5% | 0.6±0.0* ↑50% | 20.6±1.0 ↓20% | 4.4±0.2* ↑63% | 5.0±0.2 ↓12% |
| **28-HB (50µg)** | 0.9±0.0* ↓49% | 2.8±0.1 ↓18% | 0.4±0.0 ↓0% | 17.4±0.8* ↓32% | 1.9±0.1* ↓32% | 5.9±0.3 ↑3% |

*P < 0.05, as compared with control. (↑ - increase; ↓ - decrease) Values are given as Mean ± S.E.M (n=3 replicates)

ALP enzyme activity was also found increased in 10µg 28-HB treated rat liver tissue by 50%, whereas in 50µg 28-HB treated rat liver tissue, the activity was found to be same as the control. CK enzyme activity was also found increased in 50µg 28-HB treated rat kidney tissue by 5% and in 10µg 28-HB treated rat skeletal muscle tissue by 11% compared to control.

**Table 2. Effect on CK activity in rat tissue homogenates 2hr after administration of 28-HB.**

| | **Brain (IU/mg) x $10^{-3}$** | **Heart (IU/mg) x $10^{-3}$** | **Liver (IU/mg) x $10^{-3}$** | **Kidney (IU/mg) x $10^{-3}$** | **Skeletal muscle (IU/mg) x $10^{-3}$** | **Testis (IU/mg) x $10^{-3}$** |
|---|---|---|---|---|---|---|
| **Control** | 49.7±12.6 | 28.5±8.1 | 10.2±1.5 | 7.7±0.1 | 76.1±1.4 | 11.4±0.0 |
| **28-HB (10µg)** | 4.5±0.3* ↓89% | 5.0±0.3* ↓84% | 9.8±3.5 ↓4% | 5.5±1.7 ↓68% | 84.4±1.7* ↑11% | 2.2±0.2* ↓83% |
| **28-HB (50µg)** | 16.4±4.0 ↓66% | 4.6±0.5* ↓83% | 3.9±1.1* ↓65% | 8.1±2.4 ↑5% | 48.5±0.3* ↓35% | 8.8±2.5 ↓20% |

*P < 0.05, as compared with control. (↑ - increase; ↓ - decrease) Values are given as Mean ± S.E.M (n=3 replicates)

Determination of reaction rate constants for control and 28-HB treated tissue ALP and CK yielded different values (Table 3). The reaction rate constants for ALP and CK enzyme activities were in the order $10^{-7}$ mM/min for ALP and $10^{-3}$ mM/min for CK. Such differences when expressed as a percentage over the control yielded 8, 15, 20 and 35% decrease in the ALP activity of heart, kidney, testis and brain respectively due to 10µg 28-HB and 4, 15, 32 and 42% decrease in the ALP activity of heart, skeletal muscle, kidney and brain respectively, due to 50µg 28-HB.

The kinetics of ALP from kidney and testis and that of CK from heart and skeletal muscle yielded widely different Km values, while the Vmax in each case was comparable (Table 4). Time - course studies indicated decrease in both enzyme activities of control and treated rats, as a function of time. With

increase in time, ALP activity in kidney homogenate decreased by a percentage of 23, 40 and 49% in control, 10µg 28-HB and 50µg 28-HB treated rats respectively, at the end of 2hr compared to 0hr. Similarly the CK activity in skeletal muscle homogenate reduced by 12, 30 and 40% in control, 50µg 28-HB and 10µg 28-HB treated rats respectively, at the end of 2hr compared to 0hr (Table 5).

**Table 3. Reaction Rate constants of ALP and CK in Control and 28-HB treated rat tissues.**

| | ALP activity ($x10^{-4}$ µM/min) | | CK activity ($x10^{-3}$ mM/min) | |
|---|---|---|---|---|
| | **Kidney** | **Testis** | **Heart** | **Skeletal Muscle** |
| **Control** | 4.6±0.1 | 0.5±0.01 | 2.2±0.05 | 2.8±0.06 |
| **28-HB (10µg)** | 2.5±0.01 | 0.2±0.001 | 0.2±0.001 | 2.1±0.03 |
| **28-HB (50µg)** | 4.1±0.1 | 0.4±0.01 | 0.3±0.002 | 2.4±0.005 |

Values are given as Mean ± S.E.M (n=3 replicates), Rate constant (k) values are expressed in µmole.$ml^{-1}$.$min^{-1}$ for ALP activity and in mmole.$ml^{-1}$.$min^{-1}$

Total and differential cell counts estimated showed increase in RBC and WBC suggestive of erythrocytosis and leukocytosis. Increase in hemoglobin noted is to be considered due to increase in RBC numbers whereas decrease in blood glucose content was suggestive of the potential antihyperglycemic effect of 28-HB. Total serum cholesterol content was decrease by 15% compared to control following 10µg 28-HB administration, where as a 25% increase in cholesterol level was observed in serum compared to control following 28-HB (50µg) administration (Table 6).

**Table 4. Michaelis Menten Kinetics of ALP and CK enzyme activities in rat tissue homogenate 10,000 x g supernatant.**

| | Control | | 28-HB (10µg) | | 28-HB (50µg) | |
|---|---|---|---|---|---|---|
| | **Km** | **Vmax** | **Km** | **Vmax** | **Km** | **Vmax** |
| **ALP enzyme activity** | | | | | | |
| **Kidney** | 50 ±5.0 | 0.22 ±0.01 | 40 ±4.2 | 0.12 ±0.001 | 38 ±4.0 | 0.19 ±0.001 |
| **Testis** | 0.1 ±0.001 | 0.07 ±0.001 | 0.05 ±0.001 | 0.02 ±0.001 | 0.25 ±0.01 | 0.01 ±0.001 |
| **CK enzyme activity** | | | | | | |
| **Heart** | 500±25 | 0.14 ±0.001 | 2000±50 | 0.05 ±0.001 | 250±7.5 | 0.07 ±0.001 |
| **Skeletal muscle** | 1600±32 | 0.20 ±0.01 | 400±10 | 0.10 ±0.001 | 500±13 | 0.11 ±0.001 |

Km in $10^{-5}$M; Vmax in IU/ml. Values are given as Mean ± S.E.M (n=3 replicates)

## Discussion

The use of 28-HB in this study caused a reduction in the ALP and CK enzyme activities of rat brain, heart, liver, kidney, skeletal muscle and testis with few exceptions. Plasma ALP isoenzyme activities were known to change in disease condition in man. Knowledge of the conditions governing the changes was therefore important for using this enzyme as a diagnostic tool [18]. CK enzyme activity was generally assayed in blood tests as a marker for myocardial infarction (heart attack) [19]. The magnitude and direction of the changes in CK activity depended upon the disturbance in the permeability of

cell membranes [20]. CK activity is reportedly modulated by hormones in tissues containing the hormone receptors [21]. Inhibition of CK enzyme activity was suggestive of reduced creatine phosphorylation available to a cell. Since phosphor-creatine is considered an energy reservoir available to skeletal muscles for the replenishment of ATP through phosphorylation of ADP, the net energy available to a cell will be reduced by the effect of 28-HB on CK.

**Table 5. Time course studies of the effect of 28-Hb on ALP and CK enzyme activities.**

| | Enzyme activity at different time intervals | | | | |
|---|---|---|---|---|---|
| | **0** | **30** | **60** | **90** | **120** |
| **ALP activity in Kidney tissue homogenate** | | | | | |
| **Control** | 34.5±3.5 | 29.0±3.2 | 27.1±2.1 | 26.2±1.1 | 25.5±1.4 |
| **28-HB (10µg)** | | 25.0±3.1 | 24.3±3.0 | 23.6±2.8 | 20.6±1.2 |
| **28-HB (50µg)** | | 20.0±2.1 | 19.1±1.9 | 18.0±1.1 | 17.4±1.4 |
| **CK activity in Skeletal Muscle tissue homogenate** | | | | | |
| **Control** | 98.4±10.0 | 85.0±10.1 | 84.0±6.9 | 80.0±5.1 | 76.1±4.8 |
| **28-HB (10µg)** | | 94.0±9.1 | 90.0±5.6 | 86.2±5.0 | 84.4±4.5 |
| **28-HB (50µg)** | | 60.1±5.2 | 56.5±2.3 | 50.1±1.5 | 48.5±1.0 |

Enzyme activity was expressed in IU/mg $x10^{-3}$ and Time intervals in min. Values are given as Mean ± S.E.M (n=3 replicates)

The higher rate constant for CK was suggestive of a lower activity for CK in the cell than that for ALP activity. Michaelis - Menten kinetics of ALP from rat kidney and testis tissue homogenates showed reduced Km when the lower dose of 28-HB was used, indicative of probable structural changes leading to increased affinity of the enzyme for the substrate. In case of CK, the changes in Km in heart tissue among control and treated rats were not clearly understood. Further, the Km and Vmax of these enzyme activities were noted to change due to the 28-HB effect indicative of the fact that the 28-HB influence was uncompetitive in nature. Further, extensive work needs to be done in enzyme-substrate binding patterns at low dose levels to find out the reason for the changes.

**Table 6. Blood analysis**

| | **Hb (g/dl)** | **Glucose (mg/dl)** | **Cholesterol (mg/dl)** | **RBC ($x10^6$/ $mm^3$)** | **WBC** | **Neutrophils (%)** | **Lymphocytes (%)** |
|---|---|---|---|---|---|---|---|
| **Control** | 9.6 ±0.2 | 83±3.2 | 40±2.1 | 3.6±0.12 | 9500 ±50 | 63±5.0 | 35±3.0 |
| **28-HB (10µg)** | 11.0 ±0.1 | 70±1.7 | 34±1.4 | 5.0±0.05 | 9900 ±25 | 59±4.0 | 36±2.0 |
| **28-HB (50µg)** | 12.5 ±0.1 | 65±1.3 | 50±2.0 | 5.2±0.1 | 10000 ±55 | 63±5.0 | 33±1.0 |

Values are given as Mean ± S.E.M (n=3 replicates)

The observed decrease in activity of these catalytic functions with time was therefore suggestive of secondary influences within the cells that affected their rates. Clearly, 28-HB influenced the enzyme affinity for substrate as well as reaction rates *in vivo*.

Changes in RBC and WBC count, hemoglobin, blood glucose and cholesterol content pointed to the additional biological and biochemical consequence of 28-HB administration to the experimental rat. Erythrocytosis and leukocytosis developed in the

animal following the use of 28-HB suggestive of a direct involvement of the marrow for cell poiesis. Reduction in circulating glucose level due to 28-HB administration was suggestive of an antihyperglycemic factor for 28-HB. The plant oxysterol potentially acted as a glucose homeostatic agent in the rat.

## Conclusions

Variations in the ALP and CK enzyme activities taken together will reflect increase or decrease in the phosphorylation/dephosphorylation potential in the cells of a tissue, inturn affecting the state of activity and utilization of phosphorylated proteins and other phosphorylated cellular substrates. Changes will occur to the metabolic functions that utilized phosphorylated proteins and/or substrates, while potential changes in cell signaling processes that employed phosphorylated proteins can also be envisaged. Dietary phytohormones inducing such changes will have a direct influence on the animal cell metabolism, the long term consequences of which need to be studied in greater detail. Even though the physiological and pathological significance of these observations is not clear, it is suggested that the availability of low content of 28-HB in foods shall have influence on the cellular metabolic activity and that 28-HB enriched diets may not be appropriate for persons requiring higher energy for work related activities.

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